PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
GWS/MRM20873		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB 00/ 00760	02/03/2000	02/03/1999
Applicant		
MICROBIOLOGICAL RESEARCH AUTHORITY et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 5 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [X] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 2

   [X] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/00760

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N1/00 C12N1/20

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CM MCCARTHY ET AL: "Maximum growth rate of Mycobacterium avium in continuous culture or chronically infected BALB/c mice" MICROBIOS, vol. 52, 1987, pages 97-103, XP000929122 CAMBRIDGE GB page 98, paragraph 3 ---	1,4,5, 13-22
X	LG WAYNE: "Dynamics of submerged growth of Mycobacterium tuberculosis under aerobic and microaerophilic conditions" AMERICAN REVIEW OF RESPIRATORY DISEASE, vol. 114, no. 4, October 1976 (1976-10), pages 807-811, XP000915495 the whole document --- -/--	1,13,22

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 July 2000	07/08/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Cupido, M

3

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/00760

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	"DIFCO Manual: Dehydrated Culture Media and Reagents for Microbiology" 1984 , DIFCO LABORATORIES , DETROIT, USA XP002142911 pages 303 and 568	14-20

3

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N 1/00, 1/20

A1

(11) International Publication Number: WO 00/52139

(43) International Publication Date: 8 September 2000 (08.09.00)

(21) International Application Number: PCT/GB00/00760

(22) International Filing Date: 2 March 2000 (02.03.00)

(30) Priority Data:
9904773.0 2 March 1999 (02.03.99) GB

(71) Applicant (*for all designated States except US*): MICROBIOLOGICAL RESEARCH AUTHORITY [GB/GB]; CAMR, Porton Down, Salisbury SP4 0JG (GB).

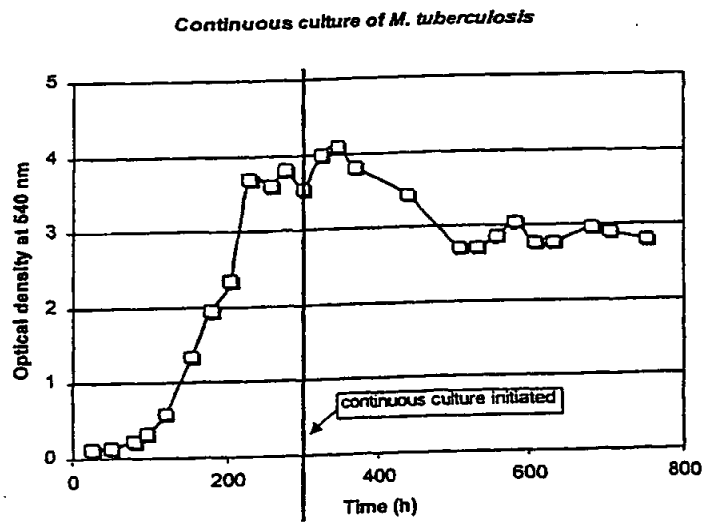
(72) Inventors; and
(75) Inventors/Applicants (*for US only*): JAMES, Brian, William [GB/GB]; Microbiological Research Authority, CAMR, Porton Down, Salisbury, Wiltshire SP4 0JG (GB). MARSH, Philip [GB/GB]; Microbiological Research Authority, CAMR, Porton Down, Salisbury, Wiltshire SP4 0JG (GB). CHADWICK, James, S. [GB/GB]; Microbiological Research Authority, CAMR, Porton Down, Salisbury, Wiltshire SP4 0JG (GB).

(74) Agents: SCHLICH, George, William et al.; Mathys & Squire, 100 Gray's Inn Road, London WC1X 8AL (GB).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: CULTURE OF MYCOBACTERIA



(57) Abstract

The present invention provides a method of culture of mycobacteria which comprises culturing the mycobacteria, either in batch or continuous culture, with agitation and in the presence of sufficient detergent so that a substantially homogenous suspension of mycobacterial cells is maintained. According to a preferred method the mycobacteria are grown in continuous culture, at a temperature of 35 °C +/– 10 °C, at a dissolved oxygen tension of at least 1 percent, at a pH of 6.9 +/– 0.9, at a dilution rate of at least 0.02 h^{-1} and with agitation in the presence of sufficient detergent to maintain a substantially homogenous suspension of single cells. The present invention also provides a growth medium for culture of mycobacteria, comprising a carbon source, a mitogen, trace elements comprising at least Mg, K, P and S, and a nitrogen source. According to a separate aspect of the invention, there is provided a method of culture of a mycobacteriophage, comprising culture of mycobacteria as described above, and contacting said mycobacteria with a mycobacteriophage.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/00760

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N1/00 C12N1/20

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CM MCCARTHY ET AL: "Maximum growth rate of Mycobacterium avium in continuous culture or chronically infected BALB/c mice" MICROBIOS, vol. 52, 1987, pages 97-103, XP000929122 CAMBRIDGE GB page 98, paragraph 3 —	1,4,5, 13-22
X	LG WAYNE: "Dynamics of submerged growth of Mycobacterium tuberculosis under aerobic and microaerophilic conditions" AMERICAN REVIEW OF RESPIRATORY DISEASE, vol. 114, no. 4, October 1976 (1976-10), pages 807-811, XP000915495 the whole document —	1,13,22

-/—

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 July 2000	07/08/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Cupido, M

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	"DIFCO Manual: Dehydrated Culture Media and Reagents for Microbiology" 1984 , DIFCO LABORATORIES , DETROIT, USA XP002142911 pages 303 and 568	14-20

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

SCHLICH, George, W.
MATHYS & SQUIRE
100 Gray's Inn Road
London WC1X 8AL
GRANDE BRETAGNE

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

	Date of mailing *(day/month/year)* 30.05.2001
Applicant's or agent's file reference GWS/MRM20873WO	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB00/00760	02/03/2000	02/03/1999

Applicant
MICROBIOLOGICAL RESEARCH AUTHORITY et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office - P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk - Pays Bas Tel. +31 70 340 - 2040 Tx: 31 651 epo nl Fax: +31 70 340 - 3016	Cardenas, C Tel.+31 70 340-3370

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
GWS/MRM20873WO		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB00/00760	02/03/2000	02/03/1999
International Patent Classification (IPC) or national classification and IPC		
C12N1/00		
Applicant		
MICROBIOLOGICAL RESEARCH AUTHORITY et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 6 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
11/08/2000	30.05.2001
Name and mailing address of the international preliminary examining authority:	Authorized officer
European Patent Office - P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk - Pays Bas Tel. +31 70 340 - 2040 Tx: 31 651 epo nl Fax: +31 70 340 - 3016	Cupido, M Telephone No. +31 70 340 3374

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB00/00760

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-3,5-7,9-25	as originally filed			
4,8	as received on	23/04/2001	with letter of	23/04/2001

Claims, No.:

1-28	as received on	23/04/2001	with letter of	23/04/2001

Drawings, sheets:

1/4-4/4	as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-28
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-28
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-28
	No:	Claims	

2. Citations and explanations
   see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

I Documents

The following documents have been taken into consideration:

D1: The DIFCO manual, 10th edition, 303 and 568 (1984)

D2: Am.Rev.Respir.Dis. **114**, 807-811 (L.G. Wayne, 1976)

D3: Microbios **52**, 97-103 (C.M. McCarthy et al., 1987)

II Novelty

In the prior art, neither methods for the culture of mycobacteria or mycobacteriophages in liquid medium and with agitation in the presence of at least 0.1% (v/v) detergent, nor a culture medium containing at least 0.1% (v/v) detergent have been disclosed. Hence, the subject-matter in claims 1-28, is novel within the meaning of Article 33(2) PCT.

III Inventive step

1. D2 is regarded as the closest prior art with respect to the question whether the novel subject-matter in the claims involves an inventive step. D2 discloses the culture of *Mycobacterium tuberculosis* in batch cultures, with agitation and in the presence of detergent. The problem to be solved by the present invention in view of D2 is the provision of further methods for the culture of mycobacteria in liquid medium.

2. The solution provided by the present application consist of an increased amount of detergent in the medium resulting in a homogenous suspension of cells. This appears to be suitable for continuous culture, which has the advantage of avoiding batch to batch variations that may be detrimental for the controlled production of mycobacterial components for therapeutic and vaccine applications. Hence the subject-matter in claims 1-22 involves an inventive step according to Article 33(3) PCT.

Re Item VIII

Certain observations on the international application

Claims 22 and 23 are referring back to the description and cannot be accepted in view of Rule 6.2(a) PCT. The exceptions to this rule as envisaged in the International Preliminary Examination Guidelines C-III, 4.10 do not apply here.

concentration of mycobacterial host available in the culture medium. Thus, a further objective of the present invention is to provide a method of culturing mycobacteriophage which overcomes/alleviates the prior art poor yield problems.

Accordingly, a first aspect of the invention provides a method of culture of mycobacteria, comprising culturing said mycobacteria, in batch or continuous culture, with agitation and in the presence of sufficient detergent so that a substantially homogenous suspension of cells is maintained.

Preferably, the method of the invention comprises growing said mycobacteria in batch or continuous culture, at a temperature of 35°C +/- 10°C, at a dissolved oxygen tension of at least 1.0 percent, at a pH of 6.9 +/- 0.9 and with agitation in the presence of sufficient detergent to maintain a substantially homogenous suspension of single cells.

In use of the present invention, illustrated by specific embodiments described below in more detail, and using *Mycobacteria tuberculosis*, we have developed a method which allows high yields of bacteria from both batch and continuous culture systems. Further, we have shown that mycobacteria generated using the methods of embodiments of the present inventions are highly virulent as demonstrated in a standard guinea pig infection model of *M.tuberculosis*. Indeed, potency of *Mycobacteria tuberculosis* grown using these methods is comparable with *M.tuberculosis* grown using the solid agar slope method.

In a specific embodiment of the present invention, growth of *M.tuberculosis* in steady-state continuous culture achieved a biomass yield of $1.2gl^{-1}$ cell dry weight. Cells grown in this continuous culture, and also in batch culture, displayed virulence comparable to cells grown on Middlebrook agar slopes, strongly indicating the suitability of these methods for growth of mycobacteria spp, such as *M. tuberculosis* or *M. bovis*, for prolonged periods in chemostat culture.

It is thus an advantage that the method of the invention enables growth at

specific embodiment a dilution rate of about 0.03 h^{-1} was achieved in continuous culture, representing a mean doubling time of about 24 hours.

The invention further provides, in a second aspect, a growth medium for culture of mycobacteria, comprising:-

a carbon source;

a mitogen;

trace elements comprising at least Mg, K, P and S;

a nitrogen source.

The carbon source is preferably selected from glucose, glycerol and an amino acid, and combinations of these carbon sources. The mitogen is present to induce cell division and is preferably asparagine, though other mitogens from inorganic sources are also suitable. Trace elements in the growth medium are preferably selected from Ca, Mg, Zn, Co, Cu, Mn, Fe, K and mixtures thereof, and the nitrogen source is selected from an amino acid and an ammonium salt.

The growth medium optionally further comprises an amino acid component selected from alanine, arginine, asparagine, aspartic acid, glutamic acid, glycine, isoleucine, leucine, phenylalanine, serine, and mixtures thereof. The amino acid component can contribute the nitrogen source in the medium.

Other optional components are a vitamin/co-factor component selected from:- inositol, thiamine, calcium pantothenate, co-enzyme A, nicotinamide, biotin, DL-thioctic acid, and mixtures thereof, preferably biotin; and one or more components selected from sodium hydroxide, glutathione, glycerol, haemin, sodium pyruvate and α-ketoglutarate, preferably glycerol and/or pyruvate.

Thus, a particularly preferred embodiment of the invention provides a method of culture of mycobacteria, comprising culturing said mycobacteria, in batch or continuous culture, with agitation in the presence of sufficient detergent so that a substantially homogenous suspension of single cells is maintained, and in the

CLAIMS

1. A method of culture of mycobacteria, comprising culturing said mycobacteria, in batch or continuous culture, with agitation and in the presence of sufficient detergent so that a substantially homogenous suspension of cells is maintained.

2. A method according to Claim 1, comprising culturing the mycobacteria in the presence of at least 0.1% (v/v) detergent.

3. A method according to Claim 1 or 2, comprising culturing the mycobacteria at a temperature of 35°C +/- 10°C.

4. A method according to any of Claims 1 to 3, comprising maintaining the pH at 6.9 +/- 0.9.

5. A method according to any of Claims 1 to 4, comprising culturing the mycobacteria with an initial dissolved oxygen concentration of at least 1% (v/v) air saturation.

6. A method according to any of Claims 1 to 5, for culture of mycobacteria selected from *M. tuberculosis*, *M. bovis* and *M. vaccae*.

7. A method according to any of Claims 1 to 6 for batch culture of mycobacteria, wherein detergent is present at from 0.1 to 1.0 % (v/v).

8. A method according to Claim 7, wherein detergent is present at about 0.2 % (v/v).

9. A method according to any of Claims 1 to 6 for continuous culture of mycobacteria, wherein detergent is present at at least 0.1 % (v/v).

10. A method according to Claim 9, wherein detergent is present at at least 0.15 % (v/v).

11. A method according to Claim 9 or 10, wherein the culture is carried out continuously with a dilution rate of at least 0.02 h^{-1}.

12. A method according to Claim 11, wherein the culture is carried out continuously with a dilution rate of at least 0.025 h^{-1}.

13. A method of culture of mycobacteria, comprising growing said mycobacteria in continuous culture, at a temperature of 35°C +/- 10°C, at a dissolved oxygen tension of at least 1 percent, at a pH of 6.9 +/- 0.9, at a dilution rate of at least 0.02 h^{-1} and with agitation in the presence of sufficient detergent to maintain a substantially homogenous suspension of single cells.

14. A growth medium for culture of mycobacteria, comprising:-
a carbon source;
a mitogen;
trace elements comprising at least Mg, K, P and S;
a nitrogen source.

15. A growth medium according to Claim 14, wherein the carbon source is selected from glucose, glycerol and an amino acid.

16. A growth medium according to Claim 14 or 15, wherein the mitogen is asparagine.

17. A growth medium according to any of Claims 14 to 16, comprising trace elements selected from Ca, Mg, Zn, Co, Cu, Mn, Fe, K, and mixtures thereof.

18. A growth medium according to any of Claims 14 to 17, wherein the nitrogen source is selected from an amino acid and an ammonium salt.

19. A growth medium according to Claim 18, comprising an amino acid component selected from alanine, arginine, asparagine, aspartic acid, glutamic acid, glycine, isoleucine, leucine, phenylalanine, serine and mixtures thereof.

20. A growth medium according to any of Claims 14 to 19, further comprising a vitamin/co-factor component selected from inositol, thiamine, calcium pantothenate, co-enzyme A, nicotinamide, biotin, DL-thioctic acid, and mixtures thereof.

21. A medium according to any of Claims 14 to 20, further comprising one or more components selected from sodium hydroxide, glutathione, glycerol, haemin, sodium pyruvate and α-ketoglutarate.

22. A method of culture of mycobacteria, comprising culturing said mycobacteria, in batch or continuous culture, with agitation in the presence of sufficient detergent so that a substantially homogenous suspension of single cells is maintained, and in the presence of a growth medium according to any of Claims 14 to 22.

23. A method of culture of mycobacteria substantially as hereinbefore described with reference to the examples.

24. A growth medium substantially as hereinbefore described with reference to the examples.

25. A method of culture of a mycobacteriophage, comprising culture of mycobacteria according to any of Claims 1-13, 22 or 23, and contacting said mycobacteria with a mycobacteriophage.

26. A method according to Claim 25, comprising challenging the mycobacteria with an agent for promoting and/or assisting mycobacteriophage adsorption on the mycobacteria.

27. A method according to Claim 25, wherein challenge occurs prior to or substantially at the same time as contacting the mycobacteria with the mycobacteriophage.

28. A method according to any of Claims 25-27, comprising reducing or minimising exposure of the phage to detergent present in the mycobacteria culture medium.

29. A method according to Claim 28, comprising allowing a phage infection to be established, and increasing the detergent concentration to an amount sufficient to maintain a substantially homogenous suspension of the mycobacterial cells.

J. Hyg., Camb. (1970), 68, 215–221

The Effect of Mechanical Agitation and CO_2 on the Growth of the BCG, $H_{37}Ra$ and RIRv Strains of *Mycobacterium tuberculosis*

MOHAMMAD NAYEBI

Department of Microbiology, The University of Tabriz, Iran

(*Received November 1969*)

SUMMARY

The application of shaker cultivation under 6% CO_2 for the production of large yields of 3 strains of tubercle bacilli was studied. There was an 18-fold increase in the yield of phenolized, washed, and dried tubercle bacilli grown on the shaker, under 6% CO_2 atmosphere, compared with unshaken incubated cultures; the increase in the yields was proportionately the same whether Tween 80 was present or absent in the medium, but the total yields were greater with Tween 80.

INTRODUCTION

A method of obtaining large yield of the $H_{37}Ra$ strain of *Mycobacterium tuberculosis* by cultivation under continuous agitation on a rotary shaker has been reported previously (Weiss, 1959). A similar enhancement of growth by incubation on a rotary shaker of BCG strain of tubercle bacilli was described 10 years ago (Lenert, Stasko & Hobby, 1958).

In our laboratory we observed an enhancement of growth of 3 strains of tubercle bacilli (BCG, $H_{37}Ra$, and RIRv), when cultures were incubated in an atmosphere of 6% CO_2 on a rotary shaker. These experiments have not been reported previously, and a brief report is made here to indicate the applicability of shaker-cultivation in an atmosphere of 6% CO_2 at 37°C for the production of a large yield of 3 strains of tubercle bacilli under conditions different from than those reported by Weiss and Lenert.

MATERIALS AND METHODS

The $H_{37}Ra$, BCG and RIRv strains of tubercle bacilli employed in these experiments have been described previously (Pierce, Dubos & Schaefer, 1953; Lenert, Stasko & Hobby, 1958; Weiss, 1959). Growth in the conventional Tween-albumin and Sauton liquid media is sparse when cultivation is stationary, with only occasional manual agitation.

The medium used in our laboratory was the standard basal mineral solution described by Dubos & Middlebrook (1947) containing, in addition to the inorganic salts, 0·3% asparagin, 0·006% ferric ammonium citrate, 0·013% sodium citrate and 1·8% bovine serum albumin fraction V (Armour). Tween 80 (Atlas) was added to some batches of this medium to a final concentration of 0·05%. The pH was adjusted to 6·8 before autoclaving. It was found not to vary by more than 0·2 pH unit after sterilization. The medium was distributed in 50 ml.

quantities in 250 ml. Erlenmeyer flasks and inoculated with 2·0 ml. aliquots of a 7-day-old culture of the organism grown in stationary culture in the same Tween containing medium. The flasks were then incubated at 37°C in an atmosphere of 6% CO_2, either with only a minute period of manual shaking daily, or on a New Brunswick gyrotory shaker at 80 rev./min. (A fan was attached to the flywheel of the shaker to dissipate the heat generated by the motor; when this was done, the temperature of the medium did not vary more than ±0·5°C from that of the stationary incubated cultures.) At intervals after incubation, the cultures were killed by the addition of liquid phenol (90%) to a final concentration of 2%. After standing in phenol for 10 hr with frequent manual shaking, the cultures of the 3 strains of tubercle bacilli were pooled separately and the bacillary mass was separated from the medium by centrifugation and washed 5 times with large quantities of cold acetone and distilled water. The mass was then dried to constant weight over anhydrous calcium chloride and weighed.

RESULTS

The results of the representative experiments are shown in Table I in which 45 flasks containing 50 ml. each of medium with and without 0·05—Tween 80 were used. 15 of these were inoculated with $H_{37}Ra$, and 15 with BCG, and the other 15 with RIRv strains of tubercle bacilli and all were incubated for 15 days; (a) without shaking; (b) without shaking and in an atmosphere of 6% CO_2; (c) on the shaker; (d) on the shaker in 6% CO_2.

It may be seen from Table I that there was an approximate 10-fold increase in the yield of phenolized, washed and dried tubercle bacilli of all 3 strains grown on the shaker, and an 18-fold increase when they were grown on the shaker in an atmosphere of 6% CO_2 but only a 2-fold increase when they were grown without shaking in an atmosphere of 6% CO_2. The increase in yields was proportionately the same whether Tween was present or absent in the medium, but the total yields were greater with Tween. The growth-enhancing effects of continuous shaking in an atmosphere of 6% CO_2 were most marked when a period of 7 to 10 days stationary incubation preceded cultivation on the shaker. Alternatively, almost equally large yields were obtained by seeding cultures very heavily. The usual procedure here was to inoculate media with one-tenth their volume of a 7 to 10 day-old Tween-albumine culture and place them on the shaker in an atmosphere of 6% CO_2. With small inocula, immediate cultivation on the shaker in an atmosphere of 6% CO_2 actually retarded growth for several days and the final yields were never as great as when large inocula were used. It thus appeared that a considerable bacillary mass must be present in cultures at the moment at which continuous agitation was begun in order to obtain a large yield of bacilli rapidly. However, even when seeding was done with relatively small inocula, of the order of one-hundredth of the volume of the production media, the yields were still consistently and considerably greater after 2 weeks or longer than were those obtained from stationary growths.

When the bacilli were cultivated on the shaker in an atmosphere of 6% CO_2 immediately after seeding with small inocula, in media containing no Tween, they usually grew in the form of discrete pellets. The bacilli of all 3 strains (BCG, $H_{37}Ra$ and RIRv) appeared to be morphologically normal, but a much greater number were found to be non-acid-fast, or only weakly acid-fast, than the bacilli grown in stationary culture.

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
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in its capacity as elected Office

Date of mailing (day/month/year) 10 October 2000 (10.10.00)	
International application No. PCT/GB00/00760	**Applicant's or agent's file reference** GWS/MRM20873
International filing date (day/month/year) 02 March 2000 (02.03.00)	**Priority date** (day/month/year) 02 March 1999 (02.03.99)
Applicant JAMES, Brian, William et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

11 August 2000 (11.08.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Olivia TEFY
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Form PCT/IB/331 (July 1992)

PATENT COOPERATION TREATY

PCT

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
GWS/MRM20873WO		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB00/00760	02/03/2000	02/03/1999

International Patent Classification (IPC) or national classification and IPC
C12N1/00

Applicant
MICROBIOLOGICAL RESEARCH AUTHORITY et al.

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 6 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
11/08/2000	30.05.2001

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB00/00760

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-3,5-7,9-25	as originally filed			
4,8	as received on	23/04/2001	with letter of	23/04/2001

Claims, No.:

1-28	as received on	23/04/2001	with letter of	23/04/2001

Drawings, sheets:

1/4-4/4	as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   ☐ the language of publication of the international application (under Rule 48.3(b)).
   ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   ☐ contained in the international application in written form.
   ☐ filed together with the international application in computer readable form.
   ☐ furnished subsequently to this Authority in written form.
   ☐ furnished subsequently to this Authority in computer readable form.
   ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims 1-28
	No:	Claims
Inventive step (IS)	Yes:	Claims 1-28
	No:	Claims
Industrial applicability (IA)	Yes:	Claims 1-28
	No:	Claims

2. Citations and explanations
   see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

I Documents

The following documents have been taken into consideration:

D1: The DIFCO manual, 10th edition, 303 and 568 (1984)

D2: Am.Rev.Respir.Dis. **114**, 807-811 (L.G. Wayne, 1976)

D3: Microbios **52**, 97-103 (C.M. McCarthy et al., 1987)

II Novelty

In the prior art, neither methods for the culture of mycobacteria or mycobacteriophages in liquid medium and with agitation in the presence of at least 0.1% (v/v) detergent, nor a culture medium containing at least 0.1% (v/v) detergent have been disclosed. Hence, the subject-matter in claims 1-28, is novel within the meaning of Article 33(2) PCT.

III Inventive step

1. D2 is regarded as the closest prior art with respect to the question whether the novel subject-matter in the claims involves an inventive step. D2 discloses the culture of *Mycobacterium tuberculosis* in batch cultures, with agitation and in the presence of detergent. The problem to be solved by the present invention in view of D2 is the provision of further methods for the culture of mycobacteria in liquid medium.

2. The solution provided by the present application consist of an increased amount of detergent in the medium resulting in a homogenous suspension of cells. This appears to be suitable for continuous culture, which has the advantage of avoiding batch to batch variations that may be detrimental for the controlled production of mycobacterial components for therapeutic and vaccine applications. Hence the subject-matter in claims 1-22 involves an inventive step according to Article 33(3) PCT.

Re Item VIII

Certain observations on the international application

Claims 22 and 23 are referring back to the description and cannot be accepted in view of Rule 6.2(a) PCT. The exceptions to this rule as envisaged in the International Preliminary Examination Guidelines C-III, 4.10 do not apply here.

concentration of mycobacterial host available in the culture medium. Thus, a further objective of the present invention is to provide a method of culturing mycobacteriophage which overcomes/alleviates the prior art poor yield problems.

Accordingly, a first aspect of the invention provides a method of culture of mycobacteria, comprising culturing said mycobacteria, in batch fermenter culture or continuous culture, with agitation and in the presence of at least 0.1% (v/v) detergent. Sufficient detergent is present so that a substantially homogenous suspension of cells is maintained.

Preferably, the method of the invention comprises growing said mycobacteria in batch fermenter culture or continuous culture, at a temperature of 35°C +/- 10°C, at a dissolved oxygen tension of at least 1.0 percent, at a pH of 6.9 +/- 0.9.

In use of the present invention, illustrated by specific embodiments described below in more detail, and using *Mycobacteria tuberculosis*, we have developed a method which allows high yields of bacteria from both batch and continuous culture systems. Further, we have shown that mycobacteria generated using the methods of embodiments of the present inventions are highly virulent as demonstrated in a standard guinea pig infection model of *M.tuberculosis*. Indeed, potency of *Mycobacteria tuberculosis* grown using these methods is comparable with *M.tuberculosis* grown using the solid agar slope method.

In a specific embodiment of the present invention, growth of *M.tuberculosis* in steady-state continuous culture achieved a biomass yield of 1.2gl^{-1} cell dry weight. Cells grown in this continuous culture, and also in batch culture, displayed virulence comparable to cells grown on Middlebrook agar slopes, strongly indicating the suitability of these methods for growth of mycobacteria spp, such as *M. tuberculosis* or *M. bovis*, for prolonged periods in chemostat culture.

It is thus an advantage that the method of the invention enables growth at

specific embodiment a dilution rate of about 0.03 h^{-1} was achieved in continuous culture, representing a mean doubling time of about 24 hours.

The invention further provides, in a second aspect, a growth medium for culture of mycobacteria, comprising:-

a carbon source;
a mitogen;
trace elements comprising at least Mg, K, P and S;
a nitrogen source; and
at least 0.1% (v/v) detergent.

The carbon source is preferably selected from glucose, glycerol and an amino acid, and combinations of these carbon sources. The mitogen is present to induce cell division and is preferably asparagine, though other mitogens from inorganic sources are also suitable. Trace elements in the growth medium are preferably selected from Ca, Mg, Zn, Co, Cu, Mn, Fe, K and mixtures thereof, and the nitrogen source is selected from an amino acid and an ammonium salt.

The growth medium optionally further comprises an amino acid component selected from alanine, arginine, asparagine, aspartic acid, glutamic acid, glycine, isoleucine, leucine, phenylalanine, serine, and mixtures thereof. The amino acid component can contribute the nitrogen source in the medium.

Other optional components are a vitamin/co-factor component selected from:- inositol, thiamine, calcium pantothenate, co-enzyme A, nicotinamide, biotin, DL-thioctic acid, and mixtures thereof, preferably biotin; and one or more components selected from sodium hydroxide, glutathione, glycerol, haemin, sodium pyruvate and α-ketoglutarate, preferably glycerol and/or pyruvate.

Thus, a particularly preferred embodiment of the invention provides a method of culture of mycobacteria, comprising culturing said mycobacteria, in batch fermenter culture or continuous culture, with agitation in the presence of at least 0.1% (v/v) detergent detergent so that a substantially homogenous suspension of single cells is maintained, and in the

CLAIMS

1. A method of culture of mycobacteria, comprising culturing said mycobacteria, in batch fermenter culture or continuous culture, with agitation and in the presence of at least 0.1% (v/v) detergent.

2. A method according to Claim 1, comprising culturing the mycobacteria at a temperature of 35°C +/- 10°C.

3. A method according to Claim 1 or 2, comprising maintaining the pH at 6.9 +/- 0.9.

4. A method according to any of Claims 1 to 3, comprising culturing the mycobacteria with an initial dissolved oxygen concentration of at least 1% (v/v) air saturation.

5. A method according to any of Claims 1 to 4, for culture of mycobacteria selected from *M. tuberculosis*, *M. bovis* and *M. vaccae*.

6. A method according to any of Claims 1 to 5 for batch culture of mycobacteria, wherein detergent is present at from 0.1 to 1.0 % (v/v).

7. A method according to Claim 6, wherein detergent is present at about 0.2 % (v/v).

8. A method according to any of Claims 1 to 5 for continuous culture of mycobacteria.

9. A method according to Claim 8, wherein detergent is present at at least 0.15 % (v/v).

10. A method according to Claim 8 or 9, wherein the culture is carried out continuously with a dilution rate of at least 0.02 h^{-1}.

11. A method according to Claim 10, wherein the culture is carried out continuously with a dilution rate of at least 0.025 h^{-1}.

12. A method according to Claim 8 or 9, comprising growing said mycobacteria in continuous culture, at a temperature of 35°C +/- 10°C, at a dissolved oxygen tension of at least 1 percent, at a pH of 6.9 +/- 0.9, at a dilution rate of at least 0.02 h^{-1}.

13. A growth medium for culture of mycobacteria, comprising:-
a carbon source;
a mitogen;
trace elements comprising at least Mg, K, P and S;
a nitrogen source; and
at least 0.1% (v/v) detergent.

14. A growth medium according to Claim 13, wherein the carbon source is selected from glucose, glycerol and an amino acid.

15. A growth medium according to Claim 13 or 14, wherein the mitogen is asparagine.

16. A growth medium according to any of Claims 13 to 15, comprising trace

elements selected from Ca, Mg, Zn, Co, Cu, Mn, Fe, K, and mixtures thereof.

17. A growth medium according to any of Claims 13 to 16, wherein the nitrogen source is selected from an amino acid and an ammonium salt.

18. A growth medium according to Claim 17, comprising an amino acid component selected from alanine, arginine, asparagine, aspartic acid, glutamic acid, glycine, isoleucine, leucine, phenylalanine, serine and mixtures thereof.

19. A growth medium according to any of Claims 13 to 18, further comprising a vitamin/co-factor component selected from inositol, thiamine, calcium pantothenate, co-enzyme A, nicotinamide, biotin, DL-thioctic acid, and mixtures thereof.

20. A medium according to any of Claims 13 to 19, further comprising one or more components selected from sodium hydroxide, glutathione, glycerol, haemin, sodium pyruvate and α-ketoglutarate.

21. A method according to any of Claims 1-12, comprising culturing said mycobacteria in the presence of a growth medium according to any of Claims 13 to 20.

22. A method of culture of mycobacteria substantially as hereinbefore described with reference to the examples.

23. A growth medium substantially as hereinbefore described with reference to the examples.

24. A method of culture of a mycobacteriophage, comprising culture of mycobacteria according to any of Claims 1-12, 21 or 22, and contacting said mycobacteria with a mycobacteriophage.

25. A method according to Claim 24, comprising challenging the mycobacteria with an agent for promoting and/or assisting mycobacteriophage adsorption on the mycobacteria.

26. A method according to Claim 24, wherein challenge occurs prior to or substantially at the same time as contacting the mycobacteria with the mycobacteriophage.

27. A method according to any of Claims 24-26, comprising reducing or minimising exposure of the phage to detergent present in the mycobacteria culture medium.

28. A method according to Claim 27, comprising allowing a phage infection to be established, and increasing the detergent concentration to at least 0.1% (v/v) detergent.